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4-Chloroacetylpyridine Adenine Dinucleotide, a Chromophoric Reagent for the Thiol Group—Study with Glutathione and with Aldehyde Dehydrogenase

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We have shown that 4-chloroacetylpyridine adenine dinucleotide (clac^{4}PdAD$^+$) inactivated the glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle by reacting with the catalytic cysteine residue 149. We observed that the modification of the thiol group gave rise to the appearance of an absorption band in the visible region, the λ_{max} depending on the pH of the medium. To test the suitability of clac^{4}PdAD$^+$ as a specific cysteine reagent we studied the modification of reduced glutathione and the mitochondrial and cytoplasmic aldehyde dehydrogenases of beef liver with clac^{4}PdAD$^+$. We found that the reaction of reduced glutathione and mitochondrial and cytosolic aldehyde dehydrogenases with clac^{4}PdAD$^+$ also gave rise to a chromophoric species whose absorption occurred at around 400 nm depending on the pH of the medium. We observed that the two modified isoenzymes had a different spectroscopic behavior at high pH values. The mitochondrial enzyme, like reduced glutathione, presented a new chromophore absorbing at 474 nm; the former was absent in the case of the cytoplasmic enzyme. This behavior and the results obtained with the glyceraldehyde-3-phosphate dehydrogenase suggest that the spectroscopic properties of the chromophore depend on the environment of the thiol group. We observed that clac^{4}PdAD$^+$ had similar inactivation properties toward aldehyde dehydrogenase isoenzymes such as disulfiram.

INTRODUCTION

Chemical modification of enzymes is a powerful technique to probe their active-site structure and to determine the enzyme mechanism. Group-specific reagents and affinity labels are used for this purpose (*1, 2*). Specific modifications of cysteine residues in proteins are well documented. Through the high nucleophilicity of the thiol group, cysteine is the most reactive amino acid and can be modified by a great number of reagents (*3, 4*). The use of chromophoric or chromogenic reagents to modify the thiol groups of a protein has allowed the cysteine content to be easily determined.

In a previous publication (*5*), we studied the inactivation of glyceraldehyde-3-phosphate dehydrogenase from sturgeon with 4-chloroacetylpyridine adenine di-

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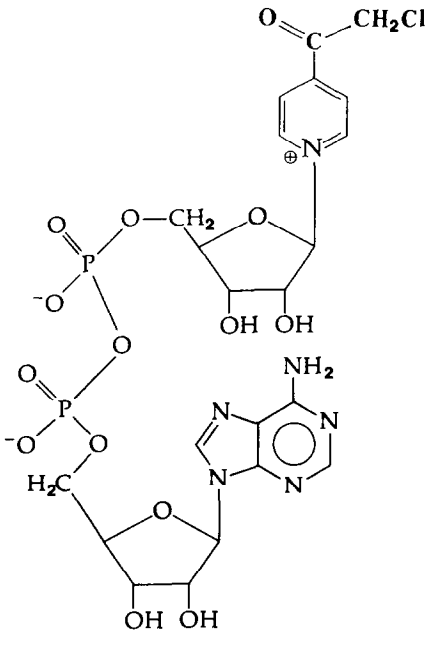

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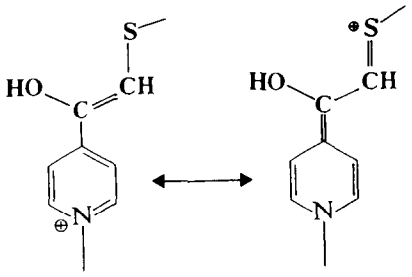
nucleotide **1** (clac^{4}PdAD$^+$)[1] which is an alkylating analog of the coenzyme NAD$^+$. The active-site thiol group of Cys-149 was shown to be modified. During the inactivation, a new absorption band appeared and its absorption was linearly related to the activity loss. In contrast to the absorption band found in the same enzyme after inactivation by reaction of the same residue with the 3-chloroacetylpyridine adenine dinucleotide (*6, 7*), the absorption was still present after denaturation. That observation suggested that the chemical nature of the covalent bond formed between the SH group and the clac^{4}PdAD$^+$ was responsible for the appearance of the absorption band. A similar long wavelength absorption band was obtained when 4-bromoacetylpyridine derivatives reacted with glyceraldehyde-3-phosphate dehydrogenase and *N*-acetylcysteine (*8–10*). The structure **2** corresponding to the enol form of an α-thioether ketone was proposed for the chromophoric group. If this proposal is correct, the absorption observed with clac^{4}PdAD$^+$-modified glyceraldehyde-3-phosphate dehydrogenase would be observed with other thiol groups. We chose to study first the reaction of clac^{4}PdAD$^+$ with a more simple molecule, glutathione, and then with another thiol enzyme, aldehyde dehydrogenase (EC 1.2.1.3) (*11*).

[1] Abbreviations and enzymes used: clac^{4}PdAD$^+$, 4-chloroacetylpyridine adenine dinucleotide; NAD$^+$, NADH, nicotinamide adenine dinucleotide oxidized and reduced forms; Tes N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; aldehyde dehydrogenase (EC 1.2.1.3); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).



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The catalytic mechanism of aldehyde dehydrogenase is thought to involve an enzyme nucleophile that forms a hemithioacetal covalent intermediate between the aldehyde and a cysteine thiol in a manner similar to that of glyceraldehyde-3-phosphate dehydrogenase (*12*). In mammalian liver, two major isoenzymes of aldehyde dehydrogenase are present and have been ascribed to the cytoplasm and mitochondria (*13*). The primary structures of aldehyde dehydrogenase isoenzymes from different mammalian liver have been published (*14–18*). They showed that the cytosolic and mitochondrial enzymes are clearly different. Comparison of the sequence reveals a greater homology between the homotopic pairs (>90%) than with the heterotopic pairs (<70%). The modification of aldehyde dehydrogenase isoenzymes from different sources with iodoacetamide or disulfiram has shown that both have a hyperreactive cysteine. This cysteine was identified as Cys-302 (*14, 19*). Cys-302 seems not to be a catalytic residue but could be localized close to the active site (*20*) or in the coenzyme binding site (*21*). We purified the mitochondrial and cytoplasmic aldehyde dehydrogenase isoenzymes of beef liver. The effect of clac^{4}PdAD$^+$ on their activity was determined.

MATERIALS AND METHODS

The reduced form of glutathione was from Fluka (Buchs, CH) and the disulfiram from Aldrich (Steinheim, FRG). The clac^{4}PdAD$^+$ preparation was given in (*5*).

Alkylation of Reduced Glutathione with clac^{4}PdAD$^+$

Glutathione (115 μM) was incubated at room temperature with clac^{4}PdAD$^+$ (115 μM) in a 25 mM Tes buffer, pH 7.1. The progress of the reaction was followed spectrophotometrically by recording absorption spectra between 350 and 550 nm.

Free thiol content was determined with DTNB (Sigma, St. Louis, MO) at 412 nm in a 0.1 M Tris buffer, pH 8.0, using an ε_{412} 13,600 M^{-1} cm^{-1} (*22*).

Influence of the pH and of the Buffer on the Absorption Band

Glutathione (3.8 mM) was alkylated with clac^{4}PdAD$^+$ (4.5 mM) in a 25 mM Tes, pH 7.1, or in a 25 mM Tris/HCl, pH 7.3, buffer. After 30 min of incubation, the

solution was diluted 76-fold. The influence of the pH was examined by adding concentrated NaOH to one-half of the solution and HCl to the other half and recording the resulting spectra. The volume increase was small (<0.5%) and was neglected.

Purification of Aldehyde Dehydrogenase Isoenzymes

Unless otherwise indicated the buffers contain 1 mM EDTA and 0.1% 2-mercaptoethanol. They were degassed and flushed with argon before use.

Bovine liver (250 g) was homogenized in a 30 mM potassium phosphate buffer, pH 6.0 (300 ml). After centrifugation (20,000 rpm, 60 min) the supernatant was treated with protamine sulfate (2 mg for 100 mg protein). After standing for 2 h the resulting precipitate was removed by centrifugation (20,000 rpm, 60 min) and discarded. The supernatant was dialyzed overnight against 30 mM potassium phosphate buffer, pH 6.0 (twice 2 liters). The enzyme solution was placed on a CM-Sephadex (Pharmacia, Sweden) column (4.4 × 27 cm) equilibrated with 30 mM potassium phosphate buffer, pH 6.0. The elution was performed with the same buffer. Fractions with aldehyde dehydrogenase activity were pooled and dialyzed against 30 mM imidazole/HCl buffer, pH 6.8. The enzyme solution was applied to a DEAE-Sephadex (Pharmacia, Sweden) column (4.4 × 27 cm) equilibrated with this buffer. Aldehyde dehydrogenase activity was eluted with a linear 0 to 0.4 M NaCl salt gradient. Enzymatically active fractions were pooled and concentrated with an Amicon ultrafiltration system (PM 10 membranes). The enzyme solution was subjected to molecular sieve chromatography on an ACA-34 Ultrogel (LKB, Sweden) column (2.4 × 85 cm) equilibrated with a 15 mM imidazole/HCl buffer, pH 7.2. The elution was performed with the same buffer. The fractions containing aldehyde dehydrogenase activity were pooled. The mitochondrial (aldehyde dehydrogenase I) and the cytoplasmic (aldehyde dehydrogenase II) isoenzymes were separated by chromatofocusing. The enzyme solution was applied to a PBE 94 (Pharmacia, Sweden) column (1.2 × 24 cm) equilibrated with a 15 mM imidazole/HCl buffer, pH 7.2, containing 1 mM DTT. The elution was performed with Polybuffer 74/HCl, pH 4.0, containing 1 mM DTT. The two isoenzymes were then precipitated by dialysis against an ammonium sulfate-saturated 20 mM Tes buffer, pH 7.0, containing 1 mM EDTA, 1 mM DTT and stored at −20°C.

Protein Estimation

The protein concentration of aldehyde dehydrogenase preparations was calculated from absorbance at 280 nm based on a $A^{1\%}_{1\,cm} = 10$. For the determination of the concentration of aldehyde dehydrogenase I and aldehyde dehydrogenase II we used $A^{1\%}_{1\,cm} = 10.2$ and $A^{1\%}_{1\,cm} = 10$ (*23*).

Enzyme Assays

Dehydrogenase activity was measured at 30°C by the absorption increase at 340 nm in sodium pyrophosphate buffer, pH 8.5, 9.65 mM (DL)-glyceraldehyde, and

1.3 mM NAD^+. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was employed for NADH.

Esterase activity was measured at 30°C by following at 400 nm the conversion of *p*-nitrophenylacetate (50 μM) to *p*-nitrophenol in a 30 mM potassium phosphate buffer, pH 7.2, using an extinction coefficient of 9.45 mM^{-1} cm^{-1}.

Alkylation of Aldehyde Dehydrogenase I and Aldehyde Dehydrogenase II with clac⁴PdAD⁺ or Disulfiram

Aldehyde dehydrogenase I (15 μM in subunit) and aldehyde dehydrogenase II (75 μM in subunit) were alkylated with a 1.2 molar ratio of clac⁴PdAD⁺ or disulfiram in a 25 mM Tes buffer, pH 7.2, at 30°C. At fixed times 10-μl aliquots were withdrawn and analyzed for residual dehydrogenase activity. To study the effect on esterase activity subunit concentrations of 75 and 140 μM for aldehyde dehydrogenase I and aldehyde dehydrogenase II were used, respectively.

Spectroscopic Properties of the clac⁴PdAD⁺-Modified Isoenzymes

Aldehyde dehydrogenase I (31 μM in subunit) was alkylated with clac⁴PdAD⁺ (26 μM) in a 25 mM Tes buffer, pH 7.2. The absorption spectra of the reaction medium were recorded between 350 and 550 nm versus time. The influence of the pH on the absorption band was studied by adding concentrated NaOH and recording the resulting spectra.

Aldehyde dehydrogenase II was alkylated with an excess and a substoichiometric amount of clac⁴PdAD⁺. The spectroscopic properties were analyzed as described for aldehyde dehydrogenase I.

Alkylation of the Disulfiram-Modified Aldehyde Dehydrogenase I Isoenzyme with clac⁴PdAD⁺

Aldehyde dehydrogenase I (20 μM in subunit) was incubated with disulfiram (40 μM) during 60 min at 30°C in a 25 mM Tes buffer, pH 7.2. After dialysis against the same buffer, the modified enzyme was incubated with a 1.2 molar ratio of clac⁴PdAD⁺. The progress of the reaction was followed spectrophotometrically and compared to the alkylation of native enzyme with clac⁴PdAD⁺.

RESULTS

Alkylation of Reduced Glutathione with clac⁴PdAD⁺

The alkylation of reduced glutathione with a stoichiometric amount of clac⁴PdAD⁺ (115 μM) in 25 mM Tes buffer, pH 7.1, leads to an absorption band centered at 398 nm increasing in intensity with incubation time. After 30 min of incubation, the residual free thiol content (TNBS) was found to be 6% of the initial value, the contribution of the absorption band at 412 nm being taken into account. An ε of 5200 M^{-1} cm^{-1} at 398 nm was calculated.

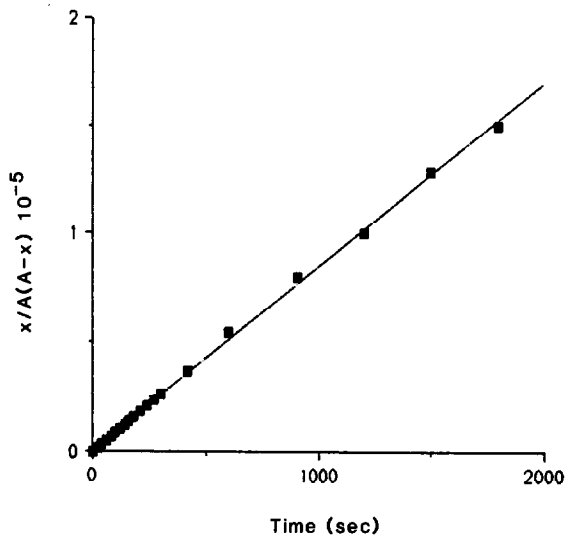

FIG. 1. Absorption increase at 398 nm with time treated as a second-order reaction of reduced glutathione (115 μM) with clac^{4}PdAD$^+$ (115 μM). x is the concentration of reaction product determined by the absorption using ε = 5200 M^{-1} cm^{-1}, A being the initial concentration of glutathione.

The reaction kinetics of glutathione with clac^{4}PdAD$^+$ agreed with a second-order reaction. A rate constant of 84 M^{-1} s^{-1} was determined (Fig. 1).

Influence of the pH on the Absorption Band

The pH of the solution was varied by successive addition of concentrated NaOH or HCl. The resulting spectra were recorded and are presented in Fig. 2 (for clearness, all spectra were not shown). The λ_{max} showed a red shift on increasing the pH (Table 1). This band was then masked by the formation of an absorption band at 474 nm. Only small variations of ε were observed. On plotting the absorbance increase at 474 nm versus pH an inflection point at pH 7.5 was observed (Fig. 3). At pH values below 7.65 no λ_{max} shift was detected and only a small increase in intensity was observed.

The alkylation of glutathione in Tris buffer also gave rise to an absorption band centered at 398 nm. An ε of 5400 M^{-1} cm^{-1} for the absorption band was determined. The pH influence on the absorption was similar to that described in Tes buffer except that the inflection point of the wavelength absorption band (λ_{max} = 474 nm) was shifted to pH 8.3.

Purification of the Mitochondrial and Cytosolic Aldehyde Dehydrogenase Isoenzymes

The two aldehyde dehydrogenase isoenzymes were isolated according to the purification scheme summarized in Table 2, each in an homogeneous state according to SDS–polyacrylamide gel electrophoresis. The chromatofocusing resolved

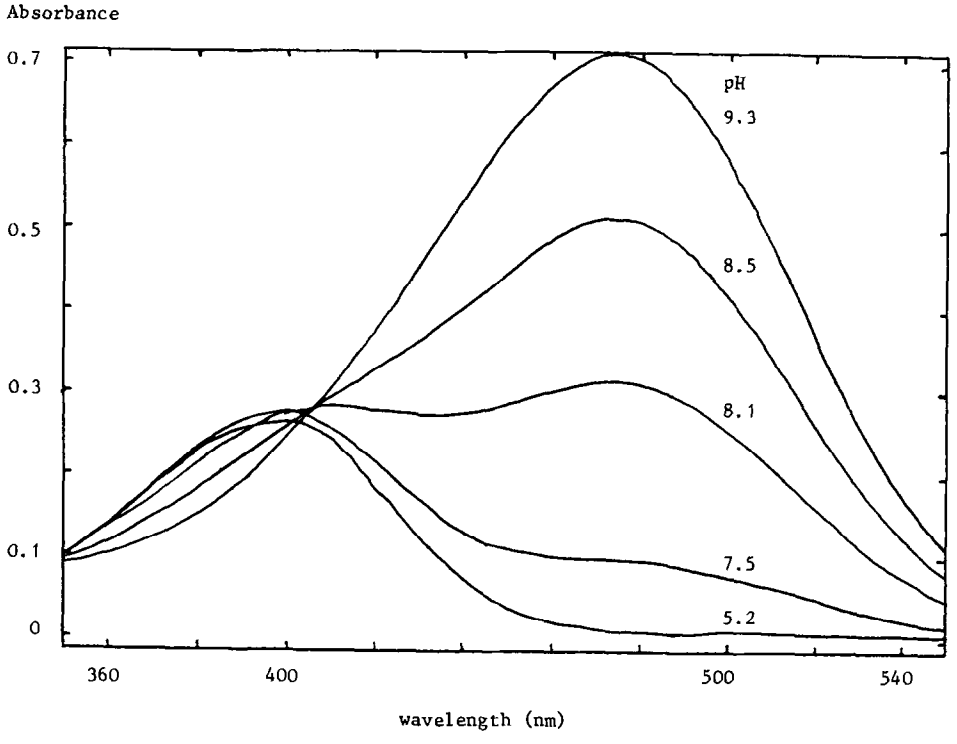

FIG. 2. Dependence on pH of the absorption spectrum of reduced glutathione (3.8 mM) reacted with clac^{4}PdAD$^+$ (4.5 mM) in a 25 mM Tes buffer, pH = 7.1. After a 76-fold dilution, the solution was adjusted to given pH with concentrated NaOH or HCl.

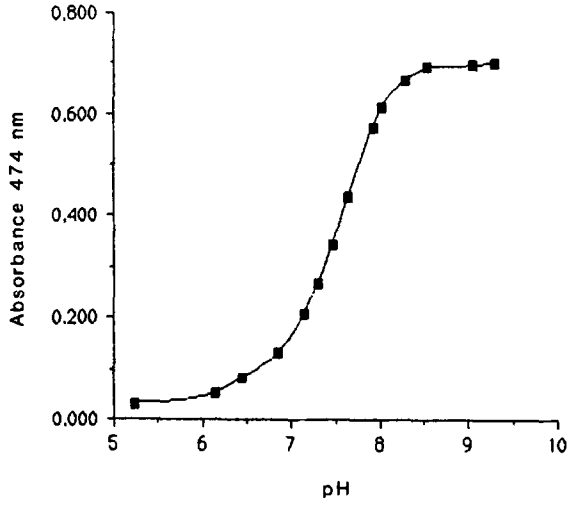

FIG. 3. pH dependence of the absorption at 474 nm of glutathione reacted with clac^{4}PdAD$^+$ (see Fig. 2).

TABLE 1

Dependence of the Maximum Absorption Wavelength and of the Absorption Coefficient of clac⁴PdAD⁺-Modified Glutathione (25 mM Tes Buffer) on pH

pH	λ_{max} (nm)	ε (M^{-1} cm^{-1})
5.2	396	5450
6.1	396	5400
6.5	396	5350
6.8	396	5250
7.0	396	5200
7.3	398	5400
7.5	398	5500
7.7	400	5650
7.9	404	5550
8.1	410	5600

the isoenzymes aldehyde dehydrogenase I and aldehyde dehydrogenase II according to their isoelectric point (Fig. 4). The fractions containing aldehyde dehydrogenase I had a pH near 6.0; aldehyde dehydrogenase II was eluted at pH 5.2–5.4. The Michaelis constants for acetaldehyde, (DL)-glyceraldehyde, and NAD^+ are reported in Table 3. One isoenzyme (aldehyde dehydrogenase I) had a very low

TABLE 2

Purification Scheme of Aldehyde Dehydrogenase Isozymes from Bovine Liver

		Protein (mg)	Specific activity (U/mg protein)[a]	Fixation degree	Total activity	Yield (%)
Liver (250 g)						
↓ Homogenization						
↓ Centrifugation						
↓ Protamine sulfate treatment		14,900	0.021	1	312.9	100
↓ CM-Sephadex chromatography		5,470	0.055	2.6	300.9	96.2
↓ DEAE-Sephadex chromatography		610	0.266	12.7	162.3	51.9
↓ Ultrogel ACA-34 filtration		164	0.981	46.7	160.9	51.4
↓ Chromatofocusing	ALDH I	45.1	1.47	70	66.3	25.7
	ALDH II	46.7	0.3		14	

[a] One unit of activity is defined as the amount of enzyme that reduces 1 μmol of NAD^+/min under the given assay conditions.

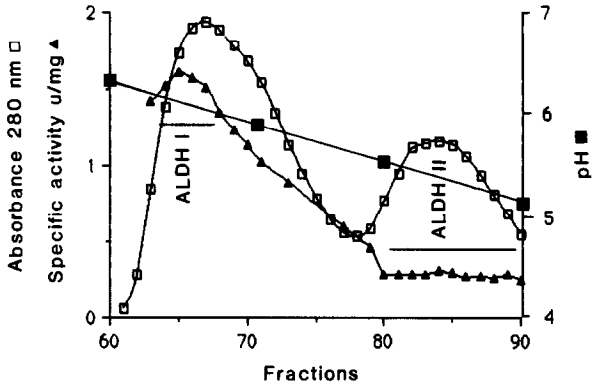

FIG. 4. Elution pattern of the protein (□) and of the aldehyde dehydrogenase activity (▲) from the chromatofocusing on a PBE 94 column according to the pH gradient (■).

K_m for acetaldehyde but a higher K_m for NAD^+ while the other isoenzyme (aldehyde dehydrogenase II) had a higher K_m for acetaldehyde and a low K_m for NAD^+. Both isoenzymes showed similar values of K_m with (DL)-glyceraldehyde as substrate. With disulfiram the behavior of aldehyde dehydrogenase I and aldehyde dehydrogenase II isoenzymes was markedly different. For aldehyde dehydrogenase I disulfiram led first to a small increase in its activity followed by a slow decrease (Fig. 5). Complete inhibition of aldehyde dehydrogenase I with disulfiram was never obtained. A 15–20% residual activity was always observed even in the presence of larger amounts of disulfiram. For aldehyde dehydrogenase II the inhibition was instantaneous and complete.

These observations allowed aldehyde dehydrogenase I to be identified as the mitochondrial isoenzyme and aldehyde dehydrogenase II was the cytosolic isoenzyme. Our data were in good agreement with those reported (*23–26*). Eckfeldt *et al.* reported that the cytosolic F1 horse liver aldehyde dehydrogenase was unsta-

TABLE 3

Michaelis Constants of NAD^+ and Substrates with Aldehyde Dehydrogenase Iosenzymes

K_m (μM)	Isoenzyme I	Isoenzyme II
NAD^+	150	15
Acetaldehyde	0.5	130
(DL)-Glyceraldehyde	230	220

Note. The activity was measured at 30°C in a sodium pyrophosphate buffer, pH 8.5. The K_m of NAD^+ was determined at saturating concentration of (DL)-glyceraldehyde (3 mM). The K_m of acetaldehyde and the K_m of (DL)-glyceraldehyde were established at saturating concentration of NAD^+ (1.8 mM for isoenzyme I, 0.18 mM for isoenzyme II).

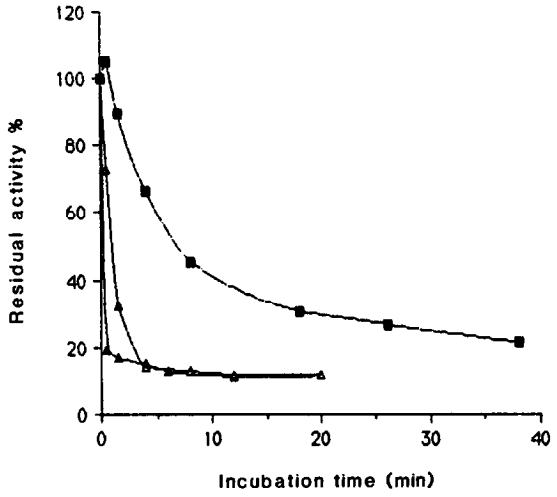

FIG. 5. Time dependence of the residual activity of aldehyde dehydrogenase isoenzyme I (15 μM in subunit) on incubation with clac^{4}PdAD$^+$ (18 μM (▲), with clac^{4}PdAD$^+$ (18 μM), in the presence of NAD$^+$ (18 μM (△), and with disulfiram (18 μM (■) in a 25 mM Tes buffer, pH 7.2.

ble when exposed to air even in the presence of EDTA and an excess of reducing agents (*27*). Aldehyde dehydrogenase II is more sensitive to oxidation than aldehyde dehydrogenase I and its low specific activity could be due to a loss of enzymatic activity caused by the oxidation of sulfhydryl groups of the native protein.

Alkylation of Aldehyde Dehydrogenase Isoenzyme I with clac^{4}PdAD$^+$

The modification of aldehyde dehydrogenase I with a 1.2 molar equivalent of clac^{4}PdAD$^+$ led to inactivation of the enzyme. The inactivation rate was greater than that observed with disulfiram (Fig. 5). As for the modification with disulfiram the inactivation was not complete even in the presence of an excess of clac^{4}PdAD$^+$. The presence of NAD$^+$ reduced the inactivation rate. The esterase activity of the enzyme was also reduced. The alkylation of aldehyde dehydrogenase I with clac^{4}PdAD$^+$ led to the formation of an absorption band centered at 408 nm. Figure 6 shows a fast optical density increase which correlates to the inactivation phase of aldehyde dehydrogenase I. Residue(s) with high reactivity and located near or in the active site must be modified during this phase. A further absorption increase occurred with no activity decrease.

To determine if clac^{4}PdAD$^+$ has modified the same amino acid as disulfiram we studied the alkylation of native and disulfiram-modified aldehyde dehydrogenase I with clac^{4}PdAD$^+$ by recording their absorption spectra between 350 and 550 nm versus time. A 20% residual activity was measured after 60 min of incubation of aldehyde dehydrogenase I with a twofold disulfiram excess. Excess reagent was eliminated by dialysis and a 1.2 molar excess of clac^{4}PdAD$^+$ was then added to the enzyme solution. We observed the formation of an absorption band centered at

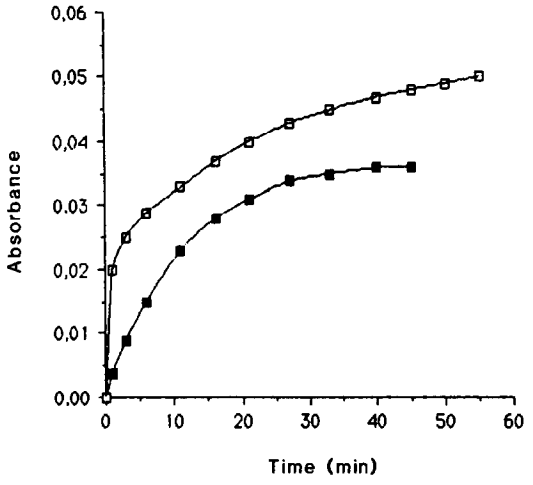

FIG. 6. Absorption increase at 410 nm of the native aldehyde dehydrogenase isoenzyme I (20 μM in subunit) modified with clac^{4}PdAD$^+$ (24 μM) (□) and at 404 nm of the disulfiram-treated isoenzyme I (20 μM in subunit) modified with clac^{4}PdAD$^+$ (24 μM) (■) in a 25 mM Tes buffer, pH 7.2, versus time. The isoenzyme I was first modified with disulfiram (40 μM) and then dialyzed to eliminate reagent excess before the reaction with clac^{4}PdAD$^+$.

404 nm while the absorption band measured with the native isoenzyme was centered at 408 nm. The alkylation of disulfiram-modified aldehyde dehydrogenase I with clac^{4}PdAD$^+$ did not show the burst (ΔOD = 0.02) observed for the native enzyme (Fig. 6). No further variation of enzyme activity was noted after clac^{4}PdAD$^+$ treatment.

To study the influence of the pH on the absorption band we alkylated aldehyde dehydrogenase I with a substoichiometric amount of clac^{4}PdAD$^+$ in order to reduce the alkylation extent of cysteine outside the active site and to have a more homogeneous modified enzyme. Table 4 and Fig. 7 show that the pH increase led

TABLE 4

pH Dependence of the Maximum Absorption Wavelength and of the Absorption Coefficient of Isoenzyme I of Aldehyde Dehydrogenase clac^{4}PdAD$^+$ Modified

pH	λ_{max} (nm)	ε
7.1	408	3.000
7.7	410	3.500
8.4	414	4.650
9.0	422	4.950
9.4	428	5.250
10.0	430	5.500
10.6	474	7.400

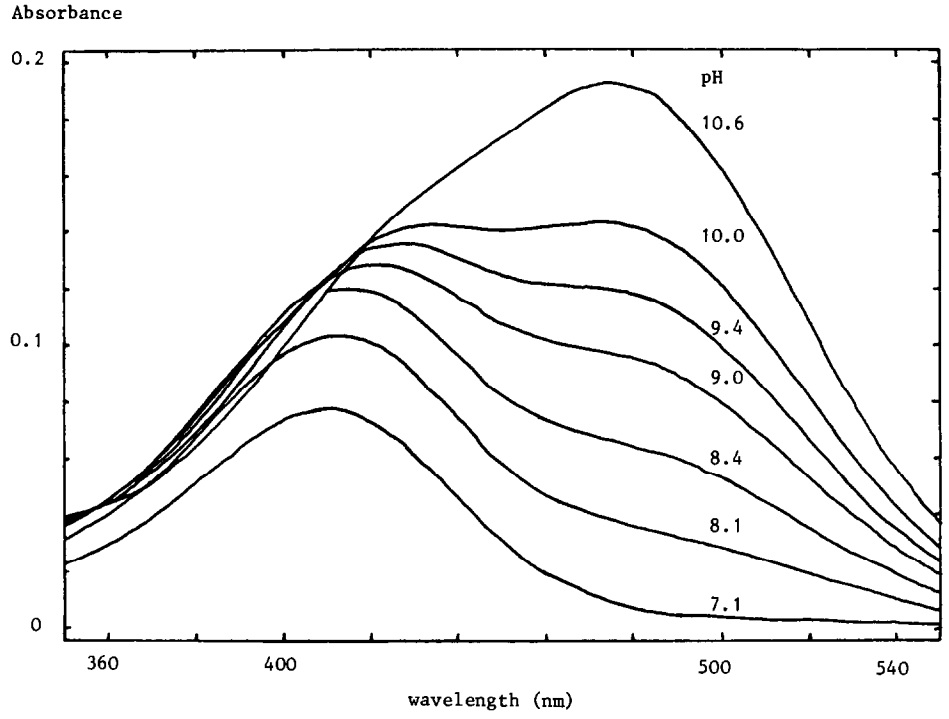

FIG. 7. pH dependence of the spectrum of aldehyde dehydrogenase isoenzyme I (31 μM in subunit) modified with clac^{4}PdAD$^+$ (26 μM) in a 25 mM Tes buffer, pH 7.2. The pH of the solution was adjusted to given pH by successive addition of concentrated NaOH.

to an increase of the absorbance and a red shift of the absorption band. In addition the formation of a new absorption band at 474 nm which is also seen for the alkylation of glutathione with clac^{4}PdAD$^+$ was observed.

Alkylation of Aldehyde Dehydrogenase Isoenzyme II with clac^{4}PdAD$^+$

The incubation of aldehyde dehydrogenase II with a 1.2 molar amount of clac^{4}PdAD$^+$ led to the complete and instantaneous inactivation of the enyzme as with disulfiram.

With isoenzyme II, the absorption maximum was centered at 410 nm. The pH dependence of the spectrum of the clac^{4}PdAD$^+$-modified isoenzyme II was much smaller than that of the modified isoenzyme I. There was only a slight absorption increase at 474 nm and the maximum wavelength absorption shifted from 410 nm a pH 7.4 to 412 nm at pH 9.0.

DISCUSSION

The reaction of the alkylating NAD$^+$ analog clac^{4}PdAD$^+$ with a low molecular weight compound, reduced glutathione, and with two isoenzymes of aldehyde

dehydrogenase was studied in order to gain knowledge about the chromophore observed in the reaction of glyceraldehyde-3-phosphate dehydrogenase with clac^{4}PdAD$^+$ (*5*) and to show the general feature of the chromophore.

The reaction of clac^{4}PdAD$^+$ with reduced glutathione could be followed directly by taking advantage of the formation of an absorption band centered at 398 nm. No appreciable deviation from second-order kinetics was observed to the end of the reaction. The alkylation of glutathione with clac^{4}PdAD$^+$ was performed at neutral pH because the reactive chloroketone group was susceptible to alkaline pH. The pH-independent rate constant (k_0 = 11,000 M^{-1} s^{-1}) was determined according to Lindley (*28*). The alkylation rate of the glutathione thiol group with clac^{4}PdAD$^+$ was much higher than that with alkyl halides (*29*). The alkylation of glutathione with iodoacetamide, for instance, is about 300-fold slower.

The appearance of the long wavelength absorption band (λ_{max} = 398 nm) when clac^{4}PdAD$^+$ reacted with reduced glutathione showed that the chemical nature of the formed covalent bond is responsible for that absorption band and not a charge transfer transition. Indeed in the case of the modification of glutathione with 3-chloroacetylpyridine adenine dinucleotide no new absorption band was detected so that the absorption band generated during the modification of glyceraldehyde-3-phosphate dehydrogenase with 3-chloroacetylpyridine adenine dinucleotide was attributed to a charge transfer transition between an amino acid of its active site and the pyridinium ring (*7*). For the chromophore generated by clac^{4}PdAD$^+$ modification we proposed the structure **2** corresponding to the enol form of an α-thioether ketone. This proposal is in line with results found with related reagents. It was found that the enol content of aryl-substituted acetophenones increases on electron withdrawal by the aromatic substituent (*30*). In our case the carbonyl group is submitted to a strong withdrawing effect of the N-alkylated pyridinium so that the enolization would be promoted. The alkylation of α-chymotrypsin at Met-192 with substituted phenacyl bromides gave rise to the formation of a new absorption band in the region 290–365 nm which was ascribed to the stabilization by the enzyme of the ylide form of the sulfonium salt produced by the alkylation of the methionine residue (*31*).

With increasing pH a new absorption band at 474 nm appeared with a 15,000 M^{-1} cm^{-1} absorption coefficient at pH 9.3. The pH dependence indicated pK_a values of 7.5 in the Tes buffer and 8.3 in the Tris buffer. The influence of the buffer nature was not obvious: Tes is a zwitterionic buffer with a pK_a of 7.5, the cationic buffer Tris has a pK_a of 8.3. A direct implication would be excluded. Charged groups on glutathione seemed not to be implicated because a similar pH-dependent absorption band was observed with clac^{4}PdAD$^+$-modified β-mercaptoethanol (results not shown). The formation of the band was reversible. The origin of this band, possibly the ionization of the enolic hydroxylic group, remains to be determined.

Two aldehyde dehydrogenase isoenzymes (I and II) of beef liver were purified to homogeneity. They correspond to the mitochondrial and cytoplasmic aldehyde dehydrogenases, respectively. The modification of both isoenzymes was performed with substoichiometric concentration of clac^{4}PdAD$^+$ to restrict the alkylation to the hyperreactive cysteine residue. clac^{4}PdAD$^+$ inactivated the two isoenzymes in a manner similar to that of disulfram: mitochondrial isoenzyme partly,

cytosolic isoenzyme completely. The modification of the disulfiram-treated aldehyde dehydrogenase I with clac^{4}PdAD$^+$ seemed to confirm that clac^{4}PdAD$^+$ reacted essentially with the same cysteine residue as disulfiram does, namely Cys-302 (*14*).

The absorption bands generated by the alkylation of glutathione and the different studied enzymes do not have the same λ_{max}. At pH 7.3 aldehyde dehydrogenase I and II gave rise to a chromophore whose absorption maxima were, respectively, 408 and 410 nm. The absorption band observed with reduced glutathione was centered at 398 nm and the chromophore formed with glyceraldehyde-3-phosphate dehydrogenase was at 430 nm (*5*). A possible explanation for these differences is that the chromophore is in an environment with different polarity. The influence of the medium polarity on related chromophores is well known. For instance, the spectra of the ylides obtained by reaction of dimethyl sulfide with substituted phenacylbromides depended on the polarity of the medium (*31*). A blue shift was observed upon increasing the polarity of the solvents. If similar considerations apply to the chromophore for the reaction of clac^{4}PdAD$^+$ with the enzyme thiol group, the chromophore produced by the alkylation of glyceraldehyde-3-phosphate dehydrogenase with clac^{4}PdAD$^+$ would be located in a more hydrophobic site than those obtained for the aldehyde dehydrogenase isoenzymes.

The behavior of the chromophore versus pH was different for the two aldehyde dehydrogenase isoenzymes. For aldehyde dehydrogenase I a new absorption band at 474 nm appeared in a manner similar to that seen with reduced glutathione and denatured glyceraldehyde-3-phosphate dehydrogenase modified with clac^{4}PdAD$^+$ (*5*). For aldehyde dehydrogenase II only a slight absorption increase at 474 nm was observed so its pH dependence was reminiscent of that observed for the clac^{4}PdAD$^+$-modified glyceraldehyde-3-phosphate dehydrogenase in nondenaturing conditions. We believe that the environment of the chromophore has an influence on this band. The geometrical constraints imposed by the protein structure would prevent the formation of the species absorbing at long wavelengths. According to our results the sites where clac^{4}PdAD$^+$ has reacted, likely Cys-302, located close to (or in) the active site of the mitochondrial and cytosolic aldehyde dehydrogenase are different. This reflects the different kinetic behavior of the two isoenzymes against clac^{4}PdAD$^+$ or disulfiram.

The fact that with the model compound and with different enzymes the generated chromophore has different spectroscopic properties raises interest in the use of clac^{4}PdAD$^+$ modification as an indicator of the environment of cysteine residues in proteins. To understand the special reactivity of functional groups in proteins, experiments to assess the polarity of their immediate environment are needed. The reactivity of a functional group at the surface of an enzyme may be different from that of an appropriate model compound in water (*2*). So the study of the spectroscopic properties of "reporter molecules" covalently bound to active-site amino acid residues has been used for probing the environment of functional groups. Such investigations have been reported for several enzymes where advantage has been taken of the difference in spectra of reporter groups depending on their ionization state (*31–34*). In our case the chromophore pro-

duced by the alkylation of thiol groups with clac^{4}PdAD$^+$ seems to have suitable spectroscopic properties for the investigation of the microenvironment of thiol functional groups in enzymes. The chromophore of the three tested enzymes showed different spectroscopic properties which would result from a different microenvironment around the thiol groups. A knowledge of the origin of the absorption bands will allow the relationship between the spectroscopic properties of the chromophore and its microenvironment to be determined. Due to its delicate synthesis, clac^{4}PdAD$^+$ seems to be not as well suited as a general specific thiol reagent. The syntheses of simpler 4-chloroacetylpyridinium derivatives are in progress. The determination of the spectroscopic properties of model compounds using these reagents will be more appropriate to investigation of the origin of the absorption bands.

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